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14. ABSTRACT  In recent years, the five-year survival rate of patients diagnosed with localized lung cancer (had not spread) is almost 50%. Although prognostic tests have been developed to evaluate the metastatic recurrence risk in lung cancer, they have shown limited success in reducing patient mortality. Clearly, there is an urgent need for tests that identify lung cancer patients at high risk of recurrence. Biomarker screens performed at the protein level hold promise in the development of such tests. In particular, it is well established that many of the differences in tumor behavior are related to protein post-translational modifications (PTMs). In this investigation, we seek to identify a small set of PTM- related, highly predictive protein biomarkers and develop test for predicting lung cancer metastatic recurrence with an emphasis on identifying high-risk patients with higher accuracy than current prognostic tests. Our technology is based on rapid separation of whole proteins from tumor biopsy samples using automated two-dimensional ultra-high pressure liquid chromatography and microfractionation to generate large protein arrays. The arrays are used as a platform to quantify the levels of specific PTMs. In this study we generated PTM-protein arrays from biopsy samples of a clinically annotated lung tumor cohort consisting of 20 patients each (recurrence < 3 years and no recurrence at 5 years of follow-up) and measured ubiquitylation levels in each array fraction (spot). Nineteen potential markers of which 10 showed increased ubiquitination in tumors and 2 had reduced protein modification. Mass spectrometry techniques identified one of the markers with increased ubiquitination as DNA methyltransferase 1 (DNMT1). These preliminary results suggest that DNMT1 may play a role in lung cancer progression and could serve as a potential biomarker for lung cancer recurrence. Further analysis of the protein arrays will likely yield additional markers.					
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## INTRODUCTION

A wealth of research shows that most lung cancers carry alterations in many post translational modification (PTM) pathways. More than 500 PTMs have been identified and the data shows that most if not all pathways in the cell are regulated by the modification of one or more of its protein components. Thereby, targeting PTMs improves the probability for identifying alterations associated with early diagnosis, risk of recurrence, treatment efficacy. Two-thirds of lung cancer patients have metastatic disease at the time of diagnosis. Even though these tumors are initially very sensitive to chemotherapy and radiotherapy they commonly become resistant and relapses are frequent. The OS of these patients at five years is around 20–25% for limited forms of the disease and 5% for disseminated forms. Therapeutic developments for the treatment of lung cancer have been disappointing. There is a pressing need for new approaches that permit a more comprehensive analysis of the molecular characteristics of tumors to aid in the assessment of prognosis and the prediction of response to various therapeutic modalities in individual patients. Our methodology overcomes several limitations of current technologies by targeting ubiquitous proteins that control the activity of most regulatory pathways, thereby providing a new avenue of research with potentially unlimited extractable data containing more relevant information that can potentially translate into a higher probability of successfully developing accurate models that predict risk of recurrence and treatment response.

## BODY

We proposed to execute a methodology recently developed in our laboratory with the goal of producing tests that predict prognosis and treatment response, to aid physicians in making more informed decisions when planning the overall therapeutic intervention of patients diagnosed with lung cancer. These new methods were used to generate large protein arrays from patient specimens that are used to quantify the levels of multiple post translational modifications (PTMs).

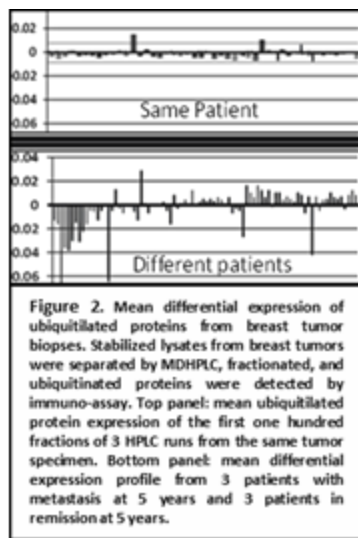
Task 1: A cohort of 250 tumor specimens derived from patients diagnosed with lung cancer was provided by our collaborator Dr. Zab Mosenifar. The specimens are fresh-frozen tumor samples that have linked data regarding patient information and biopsy immunohistochemistry. A database for the specimen cohort was constructed and saved in the Institute's server and locked to prevent any modification.

Forty samples were processed by powderizing in liquid nitrogen and aliquoting into several vials. Stable lysates were then prepared from each specimen and protein concentration was determined. These samples included 20 patients each (recurrence < 3 years and no recurrence at 5 years of follow-up).

To optimize the multi-dimensional HPLC, we first evaluated various orthogonal (different) combinations of columns including Bio-SAX (strong anion-exchange), Bio-WAX (weak anion-exchange), Bio-SCX (strong cation-exchange), and Bio-WCX (weak cation-exchange), for the first dimension. Chromatography with Bio-SAX column provided the best separation and protein recovery and was selected for HPLC in the first dimension. Effective protein separation in the second dimension was achieved using an Agilent mRP-C18 reverse phase column. To evaluate the robustness of our technology we then measured the reproducibility of the MDUHPLC platform. We generated stable lysates from lung tumor specimens enriched in ubiquitinated proteins by immunoprecipitation. The precipitates were then separated by liquid chromatography using a fully automated platform (Dionex UltiMate™ 3000 Titanium nano system, equipped with Thermostatted Column Compartment, Autosampler and micro fractionator) for ultra-high pressure and high resolution fractionation. In the first dimension, proteins were bound to an ion exchange Agilent Bio SAX column (4.6X 50 mm) and eluted with a 20mL pH gradient (pH=4 to pH=9) at a flow rate of 1 mL/min. Fractions of 2mL (10 fractions) were collected into culture tubes from each HPLC run for a total run time of 25 min. Each fraction was then separated in the second dimension using reversed phase LC (Agilent, mRP-18, 2.3X50mm). In this run, proteins were bound and then eluted with a 2 mL multi-segment gradient of acetonitrile/water in 0.1% trifluoroacetic acid at 80°C and a flow rate 0.75 mL/min. Fractions of 20uL (100 fractions) were collected into 384-well plates for a runtime of approximately 4 min. One thousand fractions were collected from each of 10 specimens in a total run time of approx. 7 hours. The variance between runs (inter) and within runs (intra) was calculated using stable lysates from the same patient tumor by methods for CLIA- certified and FDA-approved tests (28). The intra and inter-plate variability showed a high reproducibility of fractionation with all CVs less than 7%. Analysis of the fractions by denaturing gel electrophoresis (PAGE), western

blot and Coomassie staining, revealed that the eluted fractions contained between 5-40 ubiquitinated proteins with a mean of 18 proteins/spot (data not shown).

Tasks 2 and 3: To determine whether our methodology could be used to identify differential levels of PTMs in



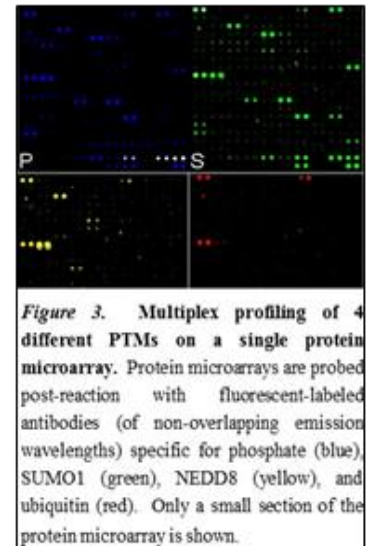
fractions from tumor lysates, we compared the levels of ubiquitinated proteins from tumors of patients with metastasis at 3 years (high risk) and patients with remission at 3 years (low risk) following diagnosis (Figure 2). The top graph shows the mean difference in the observed expression of the first 100 fractions from 3 HPLC runs using lysates from the same specimen. This graph shows the actual variability of the assay when multiple lysates derived from the same tumor specimen are separated, fractionated and ubiquitinated proteins are quantified using our methodology. Figure 2, bottom graph, shows the differential expression calculated as follows: [(mean levels of ubiquitinated proteins in each fraction of high risk patients) minus (mean levels of low risk patients)]. A positive value for any

fraction in this graph indicates higher ubiquitinated protein expression in patients with remission at 3 years. These preliminary results suggest that the PTM profiles of Lung cancer tumors with poor prognosis may be highly divergent from that of tumors from patients that were in remission at 3 years following diagnosis and these differences can be detected using our methodology.

We then focused on adapting our methodology into a multiplex format and validating the utility of our technology for biomarkers discovery. Our methods for the multiplexed assay can now detect phosphorylation, ubiquitylation, SUMO-ylation, and NEDDylation using fluorescent-labeled antibodies (Fig. 3) in protein microarrays (spotted onto coated glass slides) from breast tumor samples. Antibodies against each PTM class were labeled with a NHS-ester activated fluorescent dye of non-overlapping emission wavelength (anti-ubiquitin- Alexa Fluor 488; anti-SUMO1- Alexa Fluor 568; anti-NEDD8- Alexa Fluor 647, anti-tyrosine phosphorylation- Alexa Fluor 700) by co-incubation in PBS buffer for 30 minutes. The protein microarrays were then probed with individual fluorescent-labeled antibodies and slides read using a modified GenePix 400B Slide Scanner equipped with the appropriate detection filters (515nm/20, 620nm/50, and 670nm/20). Three experiments were performed for each antibody evaluated and assay reproducibility was assessed using validation methods for Clinical Laboratory Improvement Amendments (CLIA) or FDA approved diagnostic tests (1).

To validate the technology for identifying biomarkers associated with early tumor relapse, protein arrays were generated from a set of lung tumor specimens derived from patients in remission and patients with early relapse at 3 years (20 each). Lysates were prepared using 10 mg of tissue biopsy and ubiquitinated proteins were precipitated with 50µg anti-ubiquitin antibody (mix of 2 anti-ubiquitin Abs). The precipitates were then separated and fractionated into one 384-well plate. The levels of ubiquitinated proteins in the arrays were then measured using an optimized ELISA assay developed in our laboratory. Different antibodies were used for immunoprecipitation and ELISA detection. For improved reproducibility, the ubiquitin concentration in each fraction was calculated from a standard curve in each plate.

Nineteen of 384 fractions showed >1.5-fold mean differential (up- and down-regulated) ubiquitinated protein expression between samples from patients with early- and no-relapse. Further analysis of these fractions from all specimens was conducted by SDS PAGE and silver staining. Figure 4 shows a representative example from fractions displaying differential expression of proteins potentially associated with tumor behavior. The arrow indicates a band of approx. 250 Kd. Overexpressed in specimen from patients with tumor relapse. MALDI TOF mass spectrometry of the band excised from the stained gel of the relapse fraction (arrow) identified several unique peptides corresponding to DNA methyltransferase 1 (DNMT1) and ubiquitin. To determine whether the accumulation of DNMT1 in metastatic relapse resulted from decreased ubiquitination



(ubiq), tumor lysates were subjected to 1) immunoprecipitation with anti-human DNMT1 antibody and ubiquitin immunoblotting and 2) immunoblotting with DNMT1 antibody, and 3) immunoblotting with actin antibody. As shown in Figure 5, DNMT1 stabilization in patients with early-relapse is consistent with decreased polyubiquitination.

DNA methyltransferase I has well established roles in regulation of global DNA methylation and epigenetic silencing of tumor suppressor genes in breast and various other cancers (2). Interestingly, increased DNMT1 abundance seen in cancers appears to be largely due to dysregulation of DNMT1 protein stability rather than higher mRNA abundance (3). Increased DNMT1 protein expression has also been shown to play a critical role in the malignant progression of hepatocellular carcinoma (HCC) and be a biologic predictor of both HCC recurrence and a poor prognosis in HCC patients (4). Our preliminary results suggest that DNMT1 may also play a role in lung cancer prognosis.

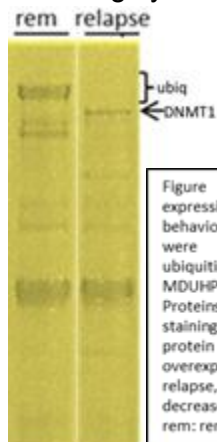


Figure 4. Differential protein expression associated with tumor behavior. Lysates from lung tumors were immunoprecipitated with ubiquitin antibody, separated by MDUHPLC and resolved by SDS PAGE. Proteins were detected by silver staining. Right arrow indicates a protein band approx. 250 Kd. overexpressed in patients with early relapse, potentially as result of decreased ubiquitination (bracket). rem: remission

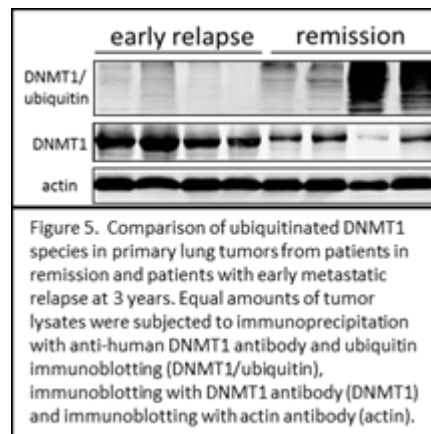


Figure 5. Comparison of ubiquitinated DNMT1 species in primary lung tumors from patients in remission and patients with early metastatic relapse at 3 years. Equal amounts of tumor lysates were subjected to immunoprecipitation with anti-human DNMT1 antibody and ubiquitin immunoblotting (DNMT1/ubiquitin), immunoblotting with DNMT1 antibody (DNMT1) and immunoblotting with actin antibody (actin).

## KEY RESEARCH ACCOMPLISHMENTS

1. Evaluation of reproducibility of multidimensional ultra-high pressure liquid fractionation and PTM detection in protein arrays.
2. PTM profiles of lung cancer tumors with poor prognosis appear to be highly divergent from that of tumors from patients that were in remission at 3 years following diagnosis and these differences can be detected using our methodology.
3. Adapted our methodology into a multiplex format that can now detect phosphorylation, ubiquitylation, SUMO-ylation, and NEDDylation using fluorescent-labeled antibodies.
4. Validated utility of our technology in biomarkers discovery for early tumor relapse.
5. Identified DNA methyltransferase 1 (DNMT1) as a potential marker of lung cancer relapse.

## REPORTABLE OUTCOMES

Nothing to report.

## CONCLUSION

We have developed a sensitive, accurate and reproducible methodology for quantifying specific PTMs at a proteomic scale, and have used this technology to identify a PTM-based biomarker predictor of lung cancer recurrence. Further analysis of the protein arrays will likely yield additional markers with the potential to identify a set of biomarkers with predictive accuracy exceeding the accuracy of existing tests. The availability of such a test in the clinic should result in more accurate prognoses and predictions for individualized decision-making and improved overall disease management for lung cancer.

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